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Cytokinesis is not controlled by calmodulin or myosin light chain kinase in the *Caenorhabditis elegans* early embryo

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Abstract Furrow ingression in animal cell cytokinesis is controlled by phosphorylation of myosin II regulatory light chain (mRLC). In *Caenorhabditis elegans* embryos, Rho-dependent Kinase (RhoK) is involved in, but not absolutely required for, this phosphorylation. The calmodulin effector myosin light chain kinase (MLCK) can also phosphorylate mRLC and is widely regarded as a candidate for redundant function with RhoK. However, our results show that RNA mediated interference against *C. elegans* calmodulin and candidate MLCKs had no effect on cytokinesis in wild-type or RhoK mutant embryos, ruling out the calmodulin/MLCK pathway as the missing regulator of cytokinesis in the *C. elegans* early embryo.

Keywords: Calmodulin; Cytokinesis; MLCK; Phosphorylation; *Caenorhabditis elegans*

1. Introduction

Phosphorylation on the regulatory light chain of myosin II (mRLC) stimulates myosin II force production thereby regulating many actomyosin based contractile events including the constriction of the contractile ring in cytokinesis [1]. Three kinases have been implicated in mRLC phosphorylation during cytokinesis; the calmodulin-dependent myosin light chain kinase (MLCK), and two RhoGTPase effector kinases: Rho-dependent kinase (RhoK) and citron kinase (CitK) [1,2]. MLCK is hypothesized to contribute to mRLC phosphorylation during cytokinesis because Ca^{2+}/calmodulin-activated MLCK is a known regulator of a similar actomyosin based contractile system, that of smooth muscle [3]. Moreover, direct evidence suggests that calmodulin and MLCK may be involved in activating the contractile ring. Both calmodulin and some isoforms of MLCK are localized to the cleavage furrow in HeLa cells [4–7]. Loss of calmodulin or application of calmodulin inhibitors disrupts or prevents cytokinesis [7,8] and a MLCK inhibitor causes cytokinesis defects in LLCPK1 cells and crane-fly spermatocytes [9,10].

Although CitK is essential for the completion of cytokinesis in proliferating tissues in *Drosophila* and in certain neuronal precursor cells in mouse [2], the most likely *Caenorhabditis elegans* CitK homologues play no role in cytokinesis [11,12]. *C. elegans* RhoK mutant embryos, on the other hand, have decreased mRLC phosphorylation and slowed division furrows that often fail. However, some mRLC phosphorylation remains and many cytokinesis events complete normally. Furthermore, double mutant embryos of RhoK and the opposing phosphatase have wild-type furrowing and levels of mRLC phosphorylation [11], suggesting that other kinases, perhaps MLCK, contribute to mRLC phosphorylation during cytokinesis.

There is as yet no non-muscle MLCK described in *C. elegans*, so we used RNA mediated interference (RNAi) to determine the role of the upstream regulator of MLCK, the calcium sensor calmodulin, as well as candidate MLCKs, during cytokinesis in early *C. elegans* embryos. We found that RNAi against calmodulin did not cause cytokinesis defects in early embryos, although subtle defects in chromosome segregation were observed. Nor were enhanced cytokinesis defects observed when calmodulin and MLCK candidates were depleted simultaneously or in the background of a RhoK mutant. These results suggest that neither calmodulin nor its effector kinases, including MLCK, regulate cytokinesis in *C. elegans* early embryos.

2. Materials and methods

2.1. C. elegans strains and alleles

The following strains were used: Bristol N2 strain (wild-type), WH0280 *unc-119(ed3);* ojEx38 [cmd-1::gfp, *unc-119(ed3)*+] (cmd-1::gfp), HR863 *let-502(sb106)* (RhoK mutant), BC3541 *dpy-18(e364)*/eT1 III; sDf52 *unc-46(e177)*/eT1[let-500(s2165)] (*cmd-1* deficiency strain), NL2099 *rrf-3(pk1426)* (RNAi sensitive strain), TY3558 (unc-119(ed3) ruIs32[pie-1::GFP::his-11] III; ojIs1[tbb-2::GFP]) (histone gfp and tubulin gfp), SU180 *itr-1(jc5)* (ITR-1 mutant), SU93 jcls1 [ajm-1::gfp] (cell junctional marker strain), and SU188 *itr-1(jc5)*; jcIs1 [ajm-1::gfp] (ITR-1 mutant crossed with the cell junctional marker strain). Standard procedures were used for the culturing and

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Abbreviations: mRLC, myosin regulatory light chain; MLCK, myosin light chain kinase; RhoK, Rho-dependent Kinase; CitK, citron kinase; RNAi, RNA mediated interference; DIC, differential interference contrast; IP_3, inositol 1,4,5 trisphosphate

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handling of strains [13]. The temperature sensitive *let-502(sb106)* mutant strain was cultured at 16 °C and shifted to 25 °C 1–2 h before imaging and the cold sensitive mutant strain *itr-1(jc5)* was cultured at 20 °C and shifted to 16 °C for 24 h before imaging.

2.2. RNA mediated interference

DNA templates for in vitro transcription of RNA (Ambion) were generated by PCR, using primers specific to the gene of interest also containing an RNA polymerase initiation site, on a cDNA clone, yk494f9 covering T21H3.3 (*cmd-1*) and genomic DNA covering the predicted open reading frame of C13C12.1 (*cal-1*) (we determined a different start site for *cal-1* than indicated on Wormbase (Supplementary data), so our PCR fragment only partially covers the predicted ORF), C18E9.1 (*cal-2*), M02B7.6 (*cal-3*) and T07G12.1 (*cal-4*). DNA templates for MLCK and CitK candidate genes were amplified from plasmids containing 1–1.5 kb of target sequence [14]. Target sequence for MLCK candidates was from W06H8.8, ZK617.1, K07A9.2, K11E8.1, K12C11.4, ZC373.4, and C09D1.1. Citron domain targets included F59A6.5, K08B12.5, T08G5.5, W02B8.2, ZC404.9, and ZC504.4a. dsRNA was injected into young adult hermaphrodites at 0.5–5 ng/μl [15].

2.3. Western blot

Embryos were dissected from 60 adults, boiled in Laemmli Buffer (BioRad) plus 5% 2-Mercaptoethanol (BioRad) 5′, and vortexed 10′ with an equal volume of 425–600 μM glass beads (Sigma). Samples were resolved by SDS–PAGE and transferred and fixed to membrane [16], then immunoblotted with antibodies at these concentrations: mouse anti-actin c4 (Sigma) 1:1333 and mouse anti-calmodulin (Zymed) at 1:333. Secondary goat anti-mouse HRP (BioRad) antibodies were detected with chemiluminescent reagents (ECL). Membranes were exposed to Biomax film (Kodak). Intensity measurements were made in Adobe Photoshop.

2.4. Live imaging

Embryos were mounted on slides and imaged with time-lapse differential interference contrast (DIC) [17]. CMD::GFP and histone::GFP; tubulin::GFP embryos were imaged by multiphoton excitation [18] and were minimally processed in Image J.

2.5. Creation of CMD-1::GFP

A full length *cmd-1* genomic DNA PCR fragment was cloned into the *pFJ1.1* plasmid (*pFJ1.1* was constructed by adding an *unc-119* rescuing fragment to the *pie-1* expression vector [19]) at the spe-1 restriction site using conventional methods and introduced into *unc-119(ed3)* worms by biolistic bombardment [20].

2.6. Embryo inhibitor studies

Embryos were exposed to calmodulin inhibitors at stages between meiosis I and II when they are still permeable to dyes and other molecules, presumably because the eggshell is not yet fully formed (unpublished observations) [21]. Embryos were dissected from adults in 3 μl inhibitor solution (calmidazolium chloride (calm) (Sigma), Compound 48/80 (c48/80) (MP Biomedicals), and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) (MP Biomedicals)) on a glass slide, covered by a cover slip suspended on a ring of petroleum jelly, and subjected to time-lapse DIC microscopy.

3. Results and discussion

3.1. Depletion of calmodulin in C. elegans embryos

There is no clear MLCK homologue in *C. elegans* yet described. To determine if the calmodulin/MLCK pathway acts in cytokinesis in *C. elegans*, we depleted the single *C. elegans* calmodulin protein in embryos. The efficiency of depletion was assessed by Western blotting using antibodies raised against *Dictyostelium* calmodulin, which recognizes bovine brain calmodulin (not shown) and *C. elegans* calmodulin in this assay (Fig. 1A). Quantification of bands, using actin as a loading control, indicated that CMD-1 was reduced by

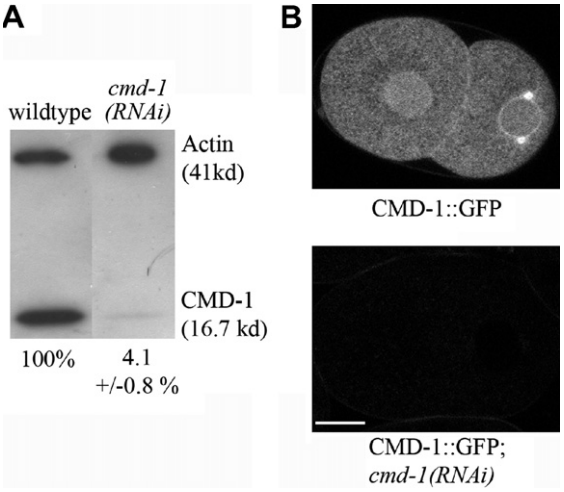

Fig. 1. CMD-1 is significantly reduced in *cmd-1* RNAi treated embryos. (A) Western Blot. Numbers indicate the percentage of calmodulin in the sample, normalized to actin. (B) Fluorescence images of two-cell embryos expressing CMD-1::GFP. Fluorescence is no longer observed in the *cmd-1(RNAi)* embryo. Bar 10 μM.

95.9 ± 0.8% in RNAi treated embryos. GFP fluorescence was also eliminated by *cmd-1(RNAi)* in embryos expressing a *cmd-1::gfp* construct (Fig. 1B).

Despite this significant reduction of CMD-1, developmental events appeared mostly normal in *cmd-1(RNAi)* early embryos (Table 1), though, as previously reported, 100% arrested at mid embryogenesis [22] (Supplementary Fig. S1). Simultaneous depletion of four calmodulin-like proteins (CAL)

Table 1
RNAi phenotypes of calmodulin and MLCK candidates

Genotype	% Cytokinesis defects in the early embryo
N2	2 (90)[a]
cmd-1(RNAi)	4 (66)
cmd-1(RNAi); cal-1(RNAi); cal-2(RNAi); cal-3(RNAi); cal-4(RNAi)[b]	3 (32)
let-502(sb106)[c]	20 (44)
let-502(sb106);cmd-1(RNAi)	30[e] (25)
let-502(sb106);ttn-1(RNAi); unc-22(RNAi);cmk-1(RNAi); unc-43(RNAi);dapk-1(RNAi); unc-89(RNAi);ZC373.4(RNAi)[d]	18[e] (>20)
itr-1(jc5)[f]	– (12)
itr-1(jc5);cmd-1(RNAi)	– (17)

[a](*N*), number of embryos scored.
[b]*cal-1-4*, calmodulin-like genes.
[c]*let502(sb106)*, RhoK loss of function mutant.
[d]*ttn-1*, *unc-22*, *cmk-1*, *unc-43*, *dapk-1*, *unc-89*, and ZC373.4, MLCK candidates. Several MLCK candidates have been described as homologues of genes other than non-muscle MLCK (see Table 2), but all were depleted to ensure that no role was missed due to functional redundancy.
[e]not significantly different from *let-502(sb106)*.
[f]*itr-1(jc5)*, IP_3R loss of function mutant.

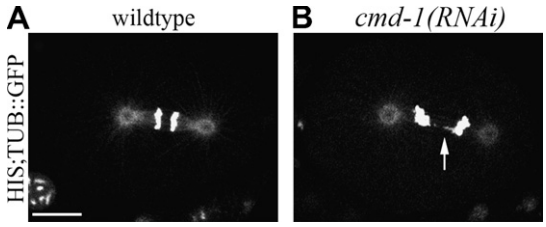

Fig. 2. Chromosome segregation is defective in *cmd-1(RNAi)* embryos. Fluorescence images of anaphase embryos expressing HIS::GFP;TUB::GFP. (A) Untreated embryo. (B) *cmd-1(RNAi)* embryo containing lagging chromosomes (arrow). Bar 10 μM.

[22,23] with CMD-1 also does not result in cytokinesis defects (Table 1).

To disrupt calmodulin further, CMD-1 was depleted by RNAi in a deficiency strain in which the chromosomal deletion sDF52 removes one copy of *cmd-1* and nearby genes, and in the RNAi sensitive mutant *rrf-3(pk1426)* [24]. In addition, embryos were treated with pharmacological calmodulin inhibitors (see Section 2). These treatments all tended to increase polar body extrusion (meiosis) failures but did not cause significant specific defects in mitotic cytokinesis (Supplementary Fig. S2).

Calmodulin, a calcium sensor, acts on many targets only when bound to calcium. Inositol 1,4,5 trisphosphate (IP_3) receptor-controlled release of calcium from the endoplasmic reticulum (ER) is the source of many cytoplasmic calcium transients [25]. A mutant of the single *C. elegans* IP_3 receptor, *itr-1(jc5)*, has defects in epithelial cell migrations during embryo enclosure but no noticeable early embryonic defects at the non-permissive temperature [26]. *itr-1(jc5)* embryos treated with *cmd-1* RNAi showed more severe and frequent cell migration defects (as assessed in embryos expressing AJM-1::GFP, a cell junction marker [27]) (Supplementary Fig. S3), but did not have cytokinesis defects (Table 1). We conclude that disruption of ITR-1-dependent calcium release does not sensitize embryos to a loss of calmodulin in the early embryo.

While calmodulin has no role in cytokinesis, we found that depletion of CMD-1 does cause subtle chromosome segregation defects in early embryos, confirming previous reports [12]. To look more closely at DNA segregation, we depleted CMD-1 in worms expressing GFP-tagged histone and tubulin. Forty-five percent of early embryo cell divisions had lagging and disorganized chromosomes, although they most often segregated successfully (Fig. 2 and Supplementary Video 1). This defect is reminiscent of that seen in yeast, where disruption of calmodulin at the spindle pole body leads to broken spindles, chromosome segregation defects, and mitotic arrest [28–31]. While *C. elegans* early embryos lacking nearly all calmodulin have grossly normal looking spindles (by light microscopy, not shown) and do not arrest, there are likely underlying spindle defects and, whereas disruption of spindle function and

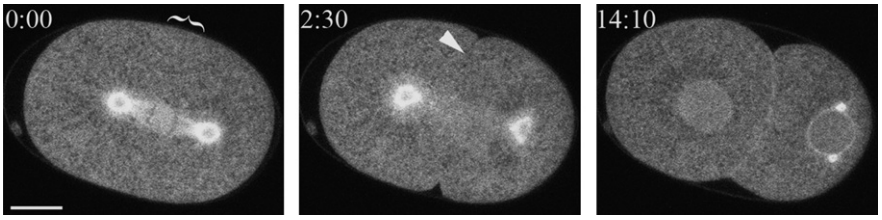

Fig. 3. CMD-1 localizes to discrete structures in *Caenorhabditis elegans* embryos. Fluorescent images of a *cmd-1::gfp* expressing embryo. 0m:00s, anaphase. 2:30, initiation of cleavage furrow ingression. 14:10, 2 cell embryo. Anterior cell (left), anaphase, posterior cell (right), interphase. CMD-1 is present at the centrosome and spindle but no accumulation is seen at the presumptive or ingressing furrow (parentheses, arrowhead). Bar 10 μM.

Table 2
Determination of *Caenorhabditis elegans* MLCK candidate proteins

Myosin light chain kinases	Isoform[a] size(s) (kDa)	IG/FN[a] Domains	Average (%) AA identity to vertebrate MLCKs (kinase domain)[a,b]	Regulatory domain[a]
Vertebrate smooth muscle/non-muscle MLCK	108, 210	0-9/1		Calmodulin
Drosophila Stretchin-MLCK short isoforms	85, 165	0-7/1		Calmodulin
Dictyostelium MLCK	33	0/0		Phosphorylation
C. elegans MLCK candidates[c]				
ttn-1 (short isoforms)	77, 80	4/0	50.5	?
unc-22	750	27/31	49.5	S100A12[d]
cmk-1	39	0/0	39.25	Calmodulin[e]
unc-43	16–80	0/0	35.25	Calmodulin[e]
dapk-1	161	0/0	42.25	?
ZC373.4	135	0/0	50.25	Calmodulin[e]
unc-89 (short isoforms)	156, 157	1/1	35.5	Calmodulin[e]

[a]Non-muscle MLCKs usually contain one or more immunoglobulin (IG) and fibronectin (FN) type III domains, are often isoforms of larger proteins, and usually contain a calmodulin binding regulatory domain in addition to a kinase domain [1,33].
[b]Average percent identity to mouse, human, and rabbit smooth muscle/non-muscle MLCK.
[c]Domain organizations of *C. elegans* candidates were determined through the NCBI conserved domain database (CCD).
[d]The S100A12 binding domain is found in a member of the S100 family of calcium binding proteins [34].
[e]The calmodulin binding domain sequence is short and not highly conserved so the stringency of blast searches was relaxed (expected *E* value threshold of 1.0) to generate these matches.

Table 3
C. elegans citron kinase candidates

C. elegans citron kinase candidates	Citron homology domain (CNH)[a]	Kinase domain[b]
F59A6.5	Yes	No
tag-59	Yes	Yes
T08G5.5	Yes	No
W02B8.2	Yes	No
gck-2	Yes	Yes
mig-15	Yes	Yes

[a]Citron kinase candidates were determined by the presence of the CNH domain (defined by Pfam).
[b]As annotated on WormBase and the NCBI conserved domain database (CDD). Note that not all CNH domain containing candidates also have kinase domains.

chromosome segregation trigger cell cycle arrest checkpoints in yeast, activation of the *C. elegans* early embryo spindle checkpoint causes only a moderate delay [32]. Further supporting these conclusions, GFP-tagged CMD-1 does not accumulate at the furrow, but does accumulate on the spindle and centrosomes (as well as to the interphase nuclear membrane and the borders of abutting cells) (Fig. 3 and Supplementary Video 2).

3.2. Calmodulin and candidate MLCKs do not function redundantly with RhoK

The *C. elegans* RhoK, LET-502, is the only kinase shown thus far to regulate myosin II activation in the furrow in *C. elegans* [11] but other kinases likely act redundantly in the regulation of this tightly controlled process. The calmodulin/MLCK pathway is not necessary for contractile ring activation in *C. elegans*, but might function redundantly with the RhoK pathway. However, when calmodulin was depleted in a RhoK mutant background, there was no enhancement of cytokinesis failures (Table 1). MLCK candidate homologues, determined by comparison of *C. elegans* peptides with the protein sequence and domain organization of known MLCKs (Table 2), do not have reported early embryonic defects when depleted in wild-type [12], and none enhance early embryonic phenotypes when depleted in pools of 3–7 in a RhoK mutant background (Table 1). Given these results, it is unlikely that a calmodulin-activated MLCK is acting in concert with RhoK to activate myosin in the contractile ring of *C. elegans* embryos.

Previous results suggested that citron kinases have no role in *C. elegans* embryo cytokinesis [11,12]. We confirmed these results and also depleted all citron domain containing proteins (Table 3) in the RhoK background but saw no effect on cytokinesis when CitK candidates were depleted simultaneously in wild-type or the RhoK mutant, although RNAi against the CitK domain containing gene F59A6.5 resulted in altered post-cleavage cortical activity (not shown).

We have shown that, unexpectedly, the Ca^{2+}/calmodulin MLCK pathway is not involved in regulation of the contractile ring in cytokinesis in *C. elegans* embryos, although calmodulin may have a modulating role in spindle organization. The missing kinase that likely acts with RhoK to phosphorylate mRLC in the contractile ring in *C. elegans* embryos must therefore be different from canonical CitKs or MLCKs that control cytokinesis in other cell types.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.08.005.

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